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Cdk5 Phosphorylation of Doublecortin Ser297 Regulates Its Effect on Neuronal Migration

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Summary

Mutations in the *doublecortin (DCX)* gene in human or targeted disruption of the *cdk5* gene in mouse lead to similar cortical lamination defects in the developing brain. Here we show that Dcx is phosphorylated by Cdk5. Dcx phosphorylation is developmentally regulated and corresponds to the timing of expression of p35, the major activating subunit for Cdk5. Mass spectrometry and Western blot analysis indicate phosphorylation at Dcx residue Ser297. Phosphorylation of Dcx lowers its affinity to microtubules in vitro, reduces its effect on polymerization, and displaces it from microtubules in cultured neurons. Mutation of Ser297 blocks the effect of Dcx on migration in a fashion similar to pharmacological inhibition of Cdk5 activity. These results suggest that Dcx phosphorylation by Cdk5 regulates its actions on migration through an effect on microtubules.

Introduction

Mammalian brain development is characterized by strictly regulated migration of each postmitotic neuron over hundreds of cell body distances from their sites of origin. The resultant cerebral cortex consists of six distinct layers, with uniform positioning of neurons of specific function. Advances in the genetic analysis of human developmental neurological disorders and genetically engineered mouse studies have identified critical genes involved in this process (Gupta et al., 2002).

In human, mutations of the *DCX* gene, located on Xq22.3-q23, lead to the severe neuronal migration disorders known as lissencephaly in males (Dobyns et al., 1996), characterized by a smooth brain surface lacking gyri and sulci, a four-layered cerebral cortex, and cerebellar hypoplasia. The 361 amino acid Dcx protein consists of an N-terminal internal repeated domain and a C-terminal serine/proline-rich domain (Sapir et al., 2000; Taylor et al., 2000). Targeted disruption of the *dcx* gene in mouse displays no significant cortical malformation (Corbo et al., 2002), possibly due to the result of functional redundancy with *dcx*-related genes, whereas acute inactivation using RNAi in developing rat neocortex led to disruption of radial migration (Bai et al., 2003). Several lines of evidences suggest it functions through regulation of microtubule dynamics. DCX colocalizes with microtubules in neurons, and overexpression leads to microtubule polymerization and stabilization (Francis et al., 1999; Gleeson et al., 1999; Horesh et al., 1999). Interaction of DCX with the μ subunits of the AP-1 adaptor protein (Friocourt et al., 2001), phospho-FIGQY tyrosine in the cytoplasmic domain of neurofascin (Kizhatil et al., 2002), and LIS1 (Caspi et al., 2000) have been identified, but how these relate to migration is unknown. Phosphorylation of DCX was suggested previously (Horesh et al., 1999), but the kinases, the substrate sites of DCX, and the effects of this phosphorylation have not yet been identified.

Cyclin-dependent kinase 5 (Cdk5) is a member of the Cdk family of serine/threonine kinases. Unlike other Cdks involved in cell cycle control, it appears to be mainly involved in phosphorylation of target proteins in terminally differentiated neurons and plays critical roles in neuronal migration, neurite outgrowth, cerebellar formation, and survival (Nikolic et al., 1996; Ohshima et al., 1996, 1999; Tanaka et al., 2001). Cdk5 is ubiquitously expressed, but its activity is strictly regulated by binding to its neuron-specific activator p35 or p39. Cdk5 phosphorylates a variety of substrates including cytoskeletal components such as NF-H, tau, or MAP1B, other kinases such as the Rac effector Pak1 (Nikolic et al., 1998), or signaling molecules such as Dab1 (Keshvara et al., 2002) and NUDEL (Niethammer et al., 2000; Sasaki et al., 2000), a protein that interacts with another lissencephaly-related protein, LIS1. Mice lacking Cdk5, p35, or both p35 and p39 display cortical lamination defects (Chae et al., 1997; Gilmore et al., 1998; Ko et al., 2001; Ohshima et al., 1996), resulting in a four-layered cortex. Cdk5 may exert its effect on neuronal migration through multiple phosphorylation substrates during brain development, but specific mechanisms are still to be elucidated.

Comparison between the cortical lamination defects caused by *DCX* mutations in human and *cdk5* deletion in mouse indicates a striking similarity. In both phenotypes, the cortex displays abnormal four layers (a normal-appearing layer 1, a cell-rich layer 2, a cell-poor layer 3, and a prominent layer 4 containing the vast majority of neurons in the cortex), instead of the normal six layers that is characteristic of the cortex in all mammals (Figure 1; Gleeson and Walsh, 2000).

Based on the similarity in phenotypes, together with evidence suggesting that Dcx is a phosphoprotein and identification of several possible Cdk5 phosphorylation sites on Dcx, we hypothesized that Dcx may serve as a substrate for Cdk5 phosphorylation. Here we show that Dcx is phosphorylated by Cdk5 in developing brain. The major site of phosphorylation of Dcx by Cdk5 was identified through site-directed mutagenesis and mass

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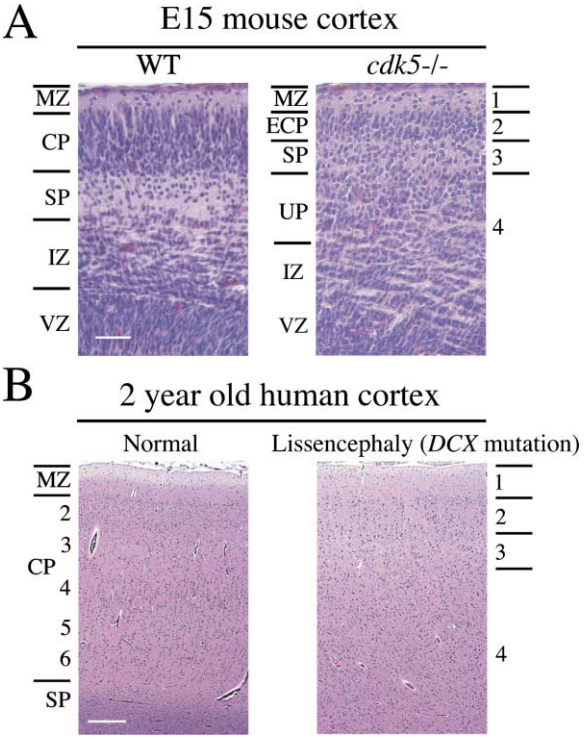


Figure 1. Phenotypic Similarity between *cdk5*$^{-/-}$ Mouse Brain and *DCX* Mutation in Human, Each Producing a Four-Layered Cortex

(A) Wild-type E15 cortex consists of marginal zone (MZ), cortical plate (CP), subplate (SP), intermediate zone (IZ), and ventricular zone (VZ). *cdk5*$^{-/-}$ brain consists of a superficial MZ layer, a thin cell-rich layer of the early cortical plate (ECP), a cell-poor SP, a thick cell-rich underplate (UP) layer that contains the majority of later-generated cortical plate neurons, and the IZ with a higher density of cells (Gilmore et al., 1998). The UP and IZ are almost fused and not clearly demarcated. Scale bar equals 200 μm.

(B) Two-year-old human cortex. Normal cortex is characterized by six well-defined layers. The lissencephaly cortex due to a *DCX* mutation contains four layers, although there is no relationship of these layers to the normal layers. Layer 1 corresponds to the molecular layer, layer 2 contains a band of mostly pyramidal neurons, layer 3 contains a cell-poor myelinated fiber network, and layer 4 contains the vast majority of cortical neurons that were presumably destined for upper layers. Scale bar equals 500 μm.

spectrometry, as residue serine 297 and a phosphopeptide-specific antibody to this site is reactive in brain lysates from wild-type but not *cdk5*$^{-/-}$ mice. Phosphorylation of Dcx by Cdk5 lowers its affinity for microtubules and reduces the microtubule polymerization effect of Dcx. Mutations of Ser297 block the normal Dcx overexpression phenotype in an in vitro neuronal migration assay. Cdk5 inhibition by roscovitine impairs the Dcx overexpression effect on migration. Together, these results suggest that phosphorylation of Dcx by Cdk5 regulates its effect on migration by reducing its microtubule binding and polymerizing effects.

Results

Dcx Is a Developmentally Regulated Phosphoprotein

Dcx was first tested for phosphorylation over several developmental time points. Western analysis of brain lysates probed with antibody created to the C-terminal fragment of Dcx (Gleeson et al., 1999) ran as a doublet, consistent with a fraction of Dcx undergoing posttranslational modification. After treatment with calf intestinal alkaline phosphatase (CIP), the slower migrating species was no longer visible, indicating the upper species represented phosphorylated Dcx (Figure 2A). A developmental profile of Dcx revealed that phosphorylated Dcx was restricted to embryonic day (E) 10 through postnatal day (P) 5, which correspond to the timing of neuronal migration in developing brain. This time window of phosphorylation is almost identical with the developmental profile of p35 expression as previously reported (Delalle et al., 1997; Patzke et al., 2003; Tomizawa et al., 1996). These data suggest that Dcx phosphorylation is developmentally regulated and corresponds to the expression of p35.

Dcx Is Codistributed with Cdk5 and p35

We next tested for codistribution of *dcx* with *p35, p39*, and *cdk5* by in situ hybridization in mouse brain at E13, E16, and E18, corresponding to the major window of neuronal migration. Strong expression of *dcx* was detected in all regions containing postmitotic migrating neurons (Figure 2B) as reported (Francis et al., 1999; Gleeson et al., 1999). There was significant expression in the developing cerebellum. Distribution of *p35* closely matched that of *dcx*. The expression of *p39* was relatively lower in the telencephalon and higher in more caudal brain, which correlates with its major site of action (Ko et al., 2001; Takahashi et al., 2003). *cdk5* showed diffuse expression with highest levels in the cortical plate, but with some expression throughout all regions of developing brain (Tsai et al., 1993). This data suggests that *dcx* is codistributed with *p35* and *cdk5* in regions containing migrating neurons in the developing brain.

In order to test for subcellular codistribution, cerebellar granule neurons were isolated from wild-type P5 mouse brains and cultured in conditions that induce migration (Hatten, 1985). Actively migrating neurons were fixed and immunostained with anti-Dcx and anti-Cdk5 or anti-p35 antibodies. Dcx was localized to microtubule structures as reported (Gleeson et al., 1999) in the perinuclear cell soma and to a lower extent in the leading and trailing processes. Strong Cdk5 immunoreactivity was observed in these same regions as reported (Matsushita et al., 1995). Although the Cdk5 signal was less fibrillar and more punctate, it was consistently contiguous with the Dcx signal (Figure 2C). Likewise, p35 immunoreactivity was observed in the same regions at the anterior perinuclear cytoplasm and to a lower extent in the leading and trailing processes. These results indicate that Dcx, Cdk5, and p35 codistribute in migrating neurons.

Previous reports indicate that a fraction of Dcx coassembles with brain microtubules (Gleeson et al., 1999; Sapir et al., 2000) and that Cdk5 displays immunoelectron microscopic localization in proximity to brain microtubules (Bhaskar et al., 2004). Therefore, we tested whether Dcx and Cdk5 co-assemble with microtubules from developing brain. The tubulin-rich fraction from E18 mouse brain was treated with taxol to generate a microtubule pellet and supernatant and these fractions

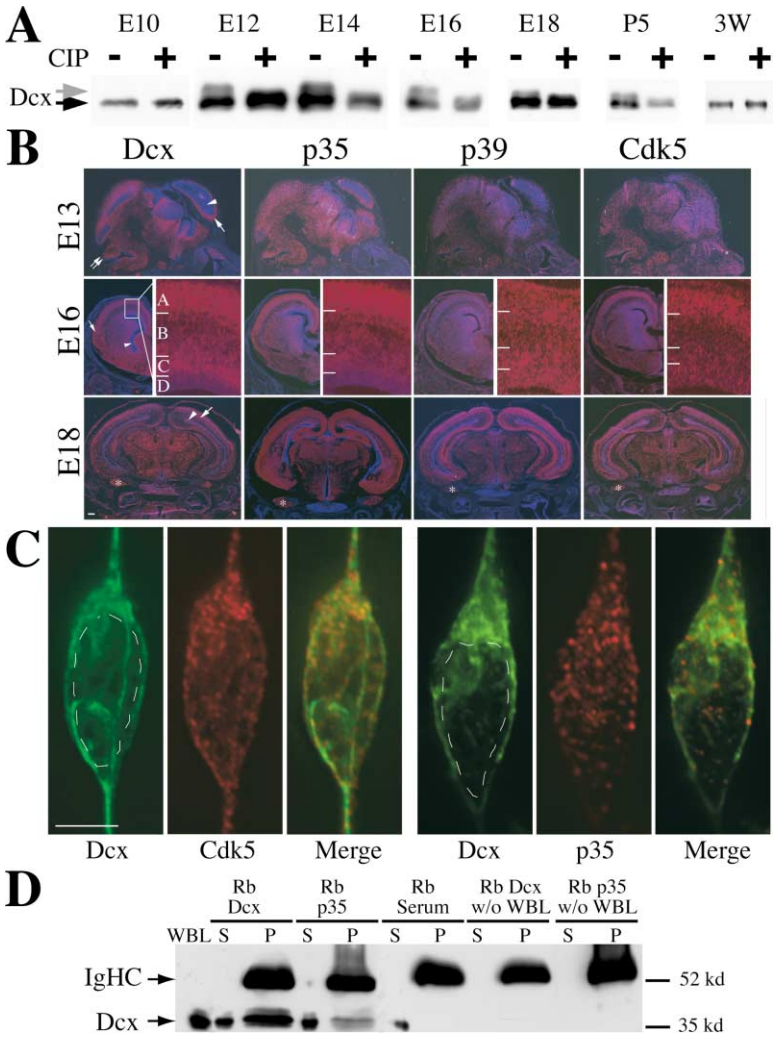


Figure 2. Phosphorylation of Dcx, Codistribution, and Co-immunoprecipitation with p35/Cdk5

(A) Dcx is a developmentally regulated phosphoprotein. Whole-brain lysates were treated with or without calf intestine alkaline phosphatase (CIP) and analyzed by Western. Dcx appears as a doublet that resolves to a single band after CIP treatment, indicating that the slower-migrating species is phosphorylated Dcx. The phosphorylated species is visible between E12 and P5.

(B) Dcx is codistributed with Cdk5 and p35 during periods of migration. In situ hybridization of *Dcx*, *p35*, *p39*, and *cdk5* (red) at E13 (sagittal), E16, and E18 (coronal) counterstained for nuclei (blue). At E16, high-power views of the outlined area are shown as inserts. A, cortical plate; B, subplate/intermediate zone; C, subventricular zone; D, ventricular zone. At all ages, expression of *Dcx* was most closely matched with *p35*, whereas *p39* and *cdk5* were more diffuse. Co-expression was noted throughout the telencephalic wall, including regions containing migrating neurons. At E13, co-expression was noted in all areas (arrow) except proliferative zones (arrowhead). At E16 there was strong codistribution in the subventricular zone and subplate region containing migrating neurons. At E18 when migration is finishing, there was some continued codistribution in the subventricular zone (arrowhead) and subplate (arrow). *cdk5* was ubiquitously expressed in all tissues analyzed, with some enrichment in neural structures, whereas *p39* had more caudal expression. Other neural structures also displayed codistribution, including the developing cerebellum (double arrow at E13) and the trigeminal nucleus (asterisk at E18). Scale bar equals 1 mm.

(C) Subcellular codistribution of Dcx with Cdk5 and p35 in migrating neurons. Dcx was localized to microtubule structures in the perinuclear cell soma. Cdk5 and p35 immunoreactivity was predominant in the same region, although staining was more punctate. Dashed circle is nuclear outline. Scale bar equals 5 μm.

(D) Dcx is part of the Cdk5/p35 complex. Co-immunoprecipitation of Dcx with p35 using lysates from E16 mouse telencephalon. Lysates were precipitated with Dcx antisera, p35 antisera, or control rabbit (Rb) sera. Additional controls included rabbit Dcx and p35 antisera, but excluded brain lysates. WBL, whole-brain lysate; S, supernatants; P, precipitates; IgHC, IgG heavy chain.

were probed for the presence of Dcx and Cdk5. Both proteins co-assembled with microtubules from brain (see Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/41/2/215/DC1), indicating that Dcx and the Cdk5 complex are both microtubule associated in brain.

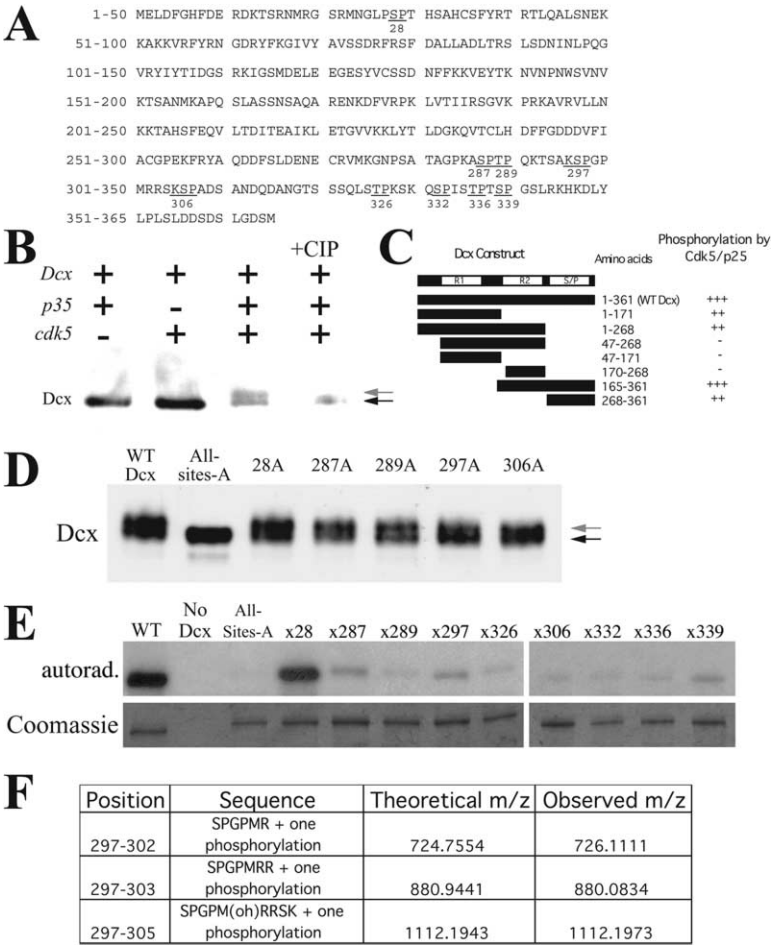
Dcx Co-immunoprecipitates with the Cdk5 Activator p35

Dcx was tested for its ability to co-immunoprecipitate with the Cdk5/p35 complex. Lysates were obtained from wild-type mouse telencephalons at E16, a time point with high Dcx phosphorylation and peak migration of cortical neurons. These were subject to immunoprecipitation with Dcx antisera, p35 antisera, or control rabbit serum. Dcx was immunoprecipitated with anti-p35 antibody, but not by control sera (Figure 2D). Similar co-immunoprecipitation results were obtained with whole-brain lysates from P5 mouse, a time point with continued migration in several brain regions (data not shown). Taken together, at least a fraction of Dcx is present in a complex with p35.

Dcx Is an In Vitro Substrate of Cdk5 Phosphorylation

The consensus sequence for Cdk5 phosphorylation was previously determined based on analysis of combinational peptide libraries as Ser (S)-Pro (P) or Thr (T)-Pro (P) motif surrounded by basic amino acids, Arg (R), Lys (K), or His (H) (Songyang et al., 1996). There are nine potential sites in Dcx that meet this criterion as possible Cdk5 substrates (Figure 3A), eight of which are found in the C-terminal serine/proline-rich domain.

In transfected 293T cells, Dcx ran as a single band on Western blot, suggesting that kinases that are active in brain are not present or not active in these cells. Therefore, we tested if *Dcx* transfected together with *cdk5/p35* could reconstitute the phosphorylation. Dcx



Position	Sequence	Theoretical m/z	Observed m/z
297-302	SPGPMR + one phosphorylation	724.7554	726.1111
297-303	SPGPMRR + one phosphorylation	880.9441	880.0834
297-305	SPGPM(oh)RRSK + one phosphorylation	1112.1943	1112.1973

Figure 3. Ser297 Is the Major Cdk5 Phosphorylation Site

(A) Dcx protein has nine possible sites for Cdk5 phosphorylation (SP motif surrounded by basic residues).

(B) Dcx is a substrate of Cdk5 phosphorylation. Lysates from 293T cells transfected with *Dcx* + *p35*, *Dcx* + *cdk5*, and *Dcx* + *cdk5/p35* were blotted with anti-Dcx antibody. Only the lysate from *Dcx* + *cdk5/p35* transfection displayed an additional slower-migrating band (gray arrow), which was removed by CIP treatment.

(C) In vitro phosphorylation of recombinant Dcx fragments with recombinant Cdk5/p25 identifies at least two major possible regions of phosphorylation, in residues 1–47 and in residues 268–361. Domains of Dcx are indicated above as R1 (first tubulin binding domain), R2 (second tubulin binding domain), and S/P (serine-proline rich tail). Relative amounts of P32 incorporation are indicated by "+," with "+++" equivalent to the incorporation in histone H1.

(D) Mutant Dcx proteins with single alanine substitution are not sufficient to block phosphorylation by Cdk5/p35. None of the single alanine substitutions of Dcx blocks phosphorylation by Cdk5/p35, whereas Dcx with all nine alanine substitutions (All-sites-A) is not phosphorylated. Black arrow indicates unphosphorylated, gray arrow indicates phosphorylated.

(E) In vitro kinase assay using Cdk5/p25 with recombinant wild-type Dcx protein, All-sites-A mutation protein, and x mutant proteins suggest phosphorylation at 28, 287, 297, and 339. The x mutant proteins have an alanine at eight of the nine sites except for one wild-type residue intact, indicated by the residue number.

(F) Tandem mass spectrometry identifies Ser297 as the major site of Cdk5/p35 phosphorylation. Ser297-containing peptide is the only peptide in MALDI-TOF analysis that shows redundant evidence (multiple molecular ions) for single phosphorylation. No other significant molecular ions that would correspond to alternative phosphorylation sites were detected using this method.

transfected both with *cdk5* and *p35* ran as a doublet on Western blot, whereas Dcx transfected with either *cdk5* or *p35* only displays a single species (Figure 3B). CIP treatment of the Dcx transfected with *cdk5/p35* removed the slower-migrating species, indicating that this corresponds to phosphorylated Dcx.

Furthermore, to exclude the possibility that *cdk5/p35* transfection activated other kinases that subsequently phosphorylate Dcx in cells, recombinant proteins were utilized. Recombinant Cdk5 and p25, which is an N-terminally truncated derivative (residues 91–307) of p35, were used in an in vitro kinase reaction with recombinant full-length or fragments corresponding to the major domains of Dcx (Taylor et al., 2000). The p25 fragment is more stable than p35 and displays constitutive binding to and activation of Cdk5 (Patrick et al., 1999). Full-length Dcx was as efficiently phosphorylated as the histone H1 positive control at equivalent concentrations, and fragments were variably phosphorylated in this assay (Figure 3C). Results indicate that there is likely to be a site of phosphorylation in amino acid 1–47 and 268–361, because Dcx fragments containing residues 1–171 or 1–268 were efficiently phosphorylated, whereas fragment containing residues 47–171 or 47–268 were not phosphorylated. These results indicate that none of the nine potential sites could be excluded using this assay.

Site-Directed Mutagenesis Suggests Phosphorylation of Dcx by Cdk5 at Several Potential Sites

In order to determine the sites of phosphorylation by Cdk5, serine or threonine in the nine potential sites were substituted with alanine by site-directed mutagenesis to create mutant Dcx proteins. Nine mutant Dcx clones were created, each of which harbored a single Ser/Thr-to-Ala mutation out of the nine potential sites, indicated by "(site number)A" alongside the "All-sites-A" mutant Dcx in which all nine sites were mutated to alanine. These were analyzed by co-transfection of 293T cells with *cdk5/p35*. All-sites-A mutant Dcx showed no slower migrating species on Western analysis, indicating that removal of all nine possible sites is sufficient to block the phosphorylation by Cdk5/p35 (Figure 3D). However, each single mutant Dcx retains the slower migrating species similar to the wild-type Dcx, suggesting that

any single mutation is not sufficient to block phosphorylation by Cdk5/p35 (Figure 3D). Similar results were obtained with many combinations of double alanine mutations (data not shown). In order to evaluate the phosphorylation state of each site individually, we then created mutant Dcx in which eight out of the nine potential sites were mutated to alanine, retaining one site intact, called "x mutants" (such as x297, which means eight sites except the 297 site are mutated). Recombinant Dcx x mutants were utilized in an in vitro kinase reaction, using wild-type Dcx as the positive control and All-sites-A mutant Dcx as the negative control. Each clone produced stable protein as shown by Coomassie staining, suggesting correct or near-correct folding. Each x mutant Dcx displayed some degree of phosphorylation, but the x28, x287, x297, and x339 displayed the highest level of phosphorylation (Figure 3E), suggesting that one or more of these may be the major site of phosphorylation by Cdk5. However, this method might not be accurate for detecting phosphorylation at adjacent residues, as accumulation of neighboring alanine mutations may alter the regional conformation of the native protein.

Serine 297 Identified as the Major Site of Cdk5/p35 Phosphorylation by MALDI MS

In order to compensate for the possible methodological problem in the previous methods, we evaluated the phosphorylation state of Dcx after incubation with active Cdk5 in vitro using mass spectrometry. Purified Dcx was phosphorylated to approximate 1 phosphate per Dcx molecule with recombinant Cdk5/p35. Tryptic digests of Dcx mixture were subjected to matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometric analysis (MS). Tryptic peptides of Dcx, Cdk5, and p35 were all taken into consideration in peptide-matching process. We found that only tryptic peptides containing phosphorylated Ser297 was detected in the MS analysis (Figure 3F). Signals from other potentially phosphorylated peptides were not detected. This suggests that Ser297 is the major phosphorylation site by Cdk5 in vitro.

Cdk5$^{-/-}$ Mice Display Differential Dcx Phosphorylation

In order to test for differential phosphorylation of Dcx by loss of Cdk5, littermates from *cdk5*$^{+/-}$ matings were collected at E17 and the brain lysates were analyzed with anti-Dcx antibody. Results demonstrated the slower-migrating species in all of the wild-type and *cdk5*$^{+/-}$ brain lysates, but either absent or significantly reduced slower-migrating species in *cdk5*$^{-/-}$ lysates (Figure 4A). Treatment of these lysates with CIP resolved these bands to a single species, indicating that the upper band represented phosphorylation. These results suggest that Cdk5 is required for proper phosphorylation of Dcx in brain development, although some phosphorylation of Dcx is present even in the absence of Cdk5.

Cdk5 Is Required for Dcx Localization to Fine Perinuclear Microtubules

In order to test for the effects of differential phosphorylation caused by *cdk5* targeted disruption on Dcx subcellular localization, cultured primary cortical neurons from *cdk5*$^{+/+}$ and *cdk5*$^{-/-}$ E17 mouse embryos were fixed and immunostained for Dcx and microtubules. In wild-type neurons, Dcx is localized to both thick microtubule bundles in proximal processes and thin microtubules in the perinuclear region. In contrast, Dcx immunostaining in *cdk5*$^{-/-}$ neurons is significantly reduced from the thin perinuclear microtubules but is retained on thick microtubule bundles in proximal processes (Figure 4B). This difference was quantitated by analyzing deconvolved images, where adequate resolution of both types of microtubules was achieved in three dimensions. The pixel intensity for tubulin and Dcx along each type of microtubule was normalized for average pixel intensity of each image. No difference was found in tubulin signal intensity either from microtubule bundles in the processes or perinuclear microtubules between *cdk5*$^{+/+}$ or *cdk5*$^{-/-}$ neurons. Additionally, no difference was found in Dcx signal intensity in the microtubule bundles in the processes. There was a significant difference found in Dcx signal intensity in perinuclear microtubules between *cdk5*$^{+/+}$ and *cdk5*$^{-/-}$ neurons (Figure 4C). Therefore, *cdk5* activity is required for proper localization of Dcx to fine perinuclear microtubules but not for localization to microtubule bundles in proximal processes.

Cdk5 Is Required for Phosphorylation of Dcx Serine 297 In Vivo

The above data suggested phosphorylation of serine 297 by Cdk5. In order to test for this, we generated an anti-phospho-serine 297 phosphopeptide-specific antibody to Dcx. The sensitivity and the specificity of this antibody was determined on Western analysis of protein from transfected cells where it specifically recognized the 297-phosphorylated form of Dcx (Figure 4D). Further, this antibody reacted against whole-brain lysates from wild-type but not *dcx* knockout E16 mice, where over 90% of the signal represented Dcx (see Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/41/2/215/DC1). These data suggest the anti-phospho-Ser297 antibody recognized phosphorylated Ser297 in Dcx specifically, with minimal cross-reactivity with other proteins.

To determine whether Cdk5 is required for phosphorylation of Ser297 in vivo, brain lysates from *cdk5*$^{+/+}$, *cdk5*$^{+/-}$, and *cdk5*$^{-/-}$ embryos at E17 were blotted with anti-phospho-Ser297 antibody. Western blot demonstrated clear bands from *cdk5*$^{+/+}$ and *cdk5*$^{+/-}$ brains, but either absent or a faint trace of band from *cdk5*$^{-/-}$ brains (Figure 4E). Additionally, none of the lysates treated with CIP showed any reactivity to the phospho-Ser297 antibody. These results suggest that Dcx Ser297 is phosphorylated predominantly by Cdk5 in vivo, although there may be partial redundancy with other kinases with significantly lower effect.

Phosphorylation of Dcx by Cdk5 Lowers Its Affinity for Microtubules In Vitro

In order to test for the effect of phosphorylation, we performed Dcx-microtubule binding (co-assembly) assays in the presence and absence of Cdk5 activity. To increase the sensitivity, increasing concentrations of NaCl were added, which facilitates the release of micro-

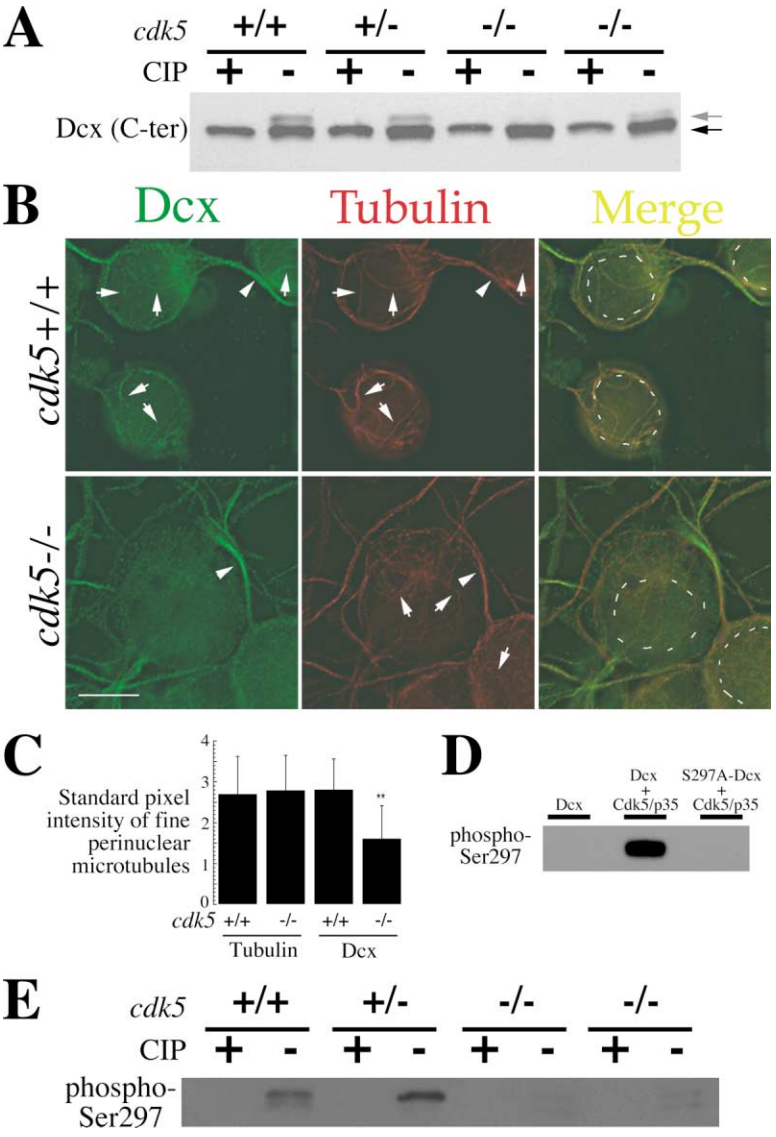


Figure 4. Cdk5 Is Required for Phosphorylation of Dcx at Residue 297

(A) Western analysis of whole-brain lysates probed with anti-Dcx antibody shows an upper (gray arrow) and lower (black arrow) band that resolve to a single band after CIP treatment, indicating phosphorylation. In *cdk5*$^{-/-}$ brain lysates, the upper band is either absent or significantly reduced in intensity.

(B) Impaired localization of Dcx in cultured primary cortical neurons from *cdk5*$^{-/-}$ E17 mouse embryo. In both *cdk5*$^{+/+}$ and *cdk5*$^{-/-}$ neurons, there are two main types of microtubules visible: thin microtubules in the perinuclear region (arrows) and thick microtubule bundles in proximal processes (arrowheads). Dcx localizes to both types of microtubules in *cdk5*$^{+/+}$ neurons. In *cdk5*$^{-/-}$ neurons, Dcx localizes to thick microtubules but is significantly reduced from thin microtubules. Nucleus indicated by dashed circle. Scale bar equals 10 μm.

(C) Quantitation of pixel intensity of fine perinuclear microtubules from (B), normalized by dividing by the average pixel intensity of the image, demonstrated reduced Dcx signal from *cdk5*$^{-/-}$ neurons. **p $<$ 0.05, Student's t test.

(D) Phospho-specific antibody raised against phospho-serine at residue 297 (phospho-Ser297-Dcx) recognizes a band in 293T cell lysate transfected with Dcx + Cdk5/p35. The antibody does not recognize Dcx without Cdk5/p35, nor S297A mutant Dcx with Cdk5/p35.

(E) Phospho-serine297 antibody is reactive in brain lysates from wild-type mice but only barely detectable in *cdk5*$^{-/-}$ mice. No reactivity was detected following CIP treatment.

tubule-associated proteins from microtubules (Vallee, 1986). Recombinant Dcx was treated with recombinant Cdk5/p25 to allow for phosphorylation. Cdk5 and p25 were then removed from the reaction, and Dcx was incubated with tubulin plus taxol, with 0 M, 0.1 M, 0.25 M, or 0.35 M NaCl, then centrifuged to produce pellet and supernatant fractions. In the absence of Cdk5 activity, Dcx was detected exclusively in the microtubule pellet under the conditions of 0 M and 0.1 M of NaCl, whereas some Dcx was detected in the supernatant at 0.25 M NaCl (Figure 5A). In the presence of Cdk5 activity, Dcx was more readily released from microtubules at 0.1 M NaCl, and a greater proportion was released at each stepwise increase in NaCl concentration. These data suggest that phosphorylation by Cdk5 lowers the affinity of Dcx to microtubules in vitro.

Phosphorylation of Dcx by Cdk5 Reduces Its Effect on Microtubule Polymerization

The impaired binding of Cdk5-phosphorylated Dcx to microtubules may attenuate its effect on microtubule polymerization. In order to test this, a turbidimetric microtubule polymerization assay was performed using Dcx treated with either active Cdk5/p25 or heat-inactivated Cdk5/p25. The experiment with each condition was repeated three times and the average intensity of turbidity was plotted (Figure 5B). Dcx treated with heat-inactivated Cdk5/p25 led to rapid microtubule polymerization similar to Dcx alone (Gleeson et al., 1999; Horesh et al., 1999; Taylor et al., 2000). In contrast, the same amount of Dcx treated with Cdk5/p25 led to slower microtubule polymerization rates and averaged 60% of the signal without Cdk5 activity at each time point. Active or heat-inactivated Cdk5/p25 alone had no effect on tubulin polymerization ratio (not shown). This data indicates Dcx phosphorylated by Cdk5 has reduced effect on microtubule polymerization compared with unphosphorylated Dcx.

In the previous experiment, Dcx may have been phosphorylated at multiple residues by Cdk5, and so to test for the specific effect of phosphorylation of Dcx at residue 297, a pseudo-phosphorylation mutant was created

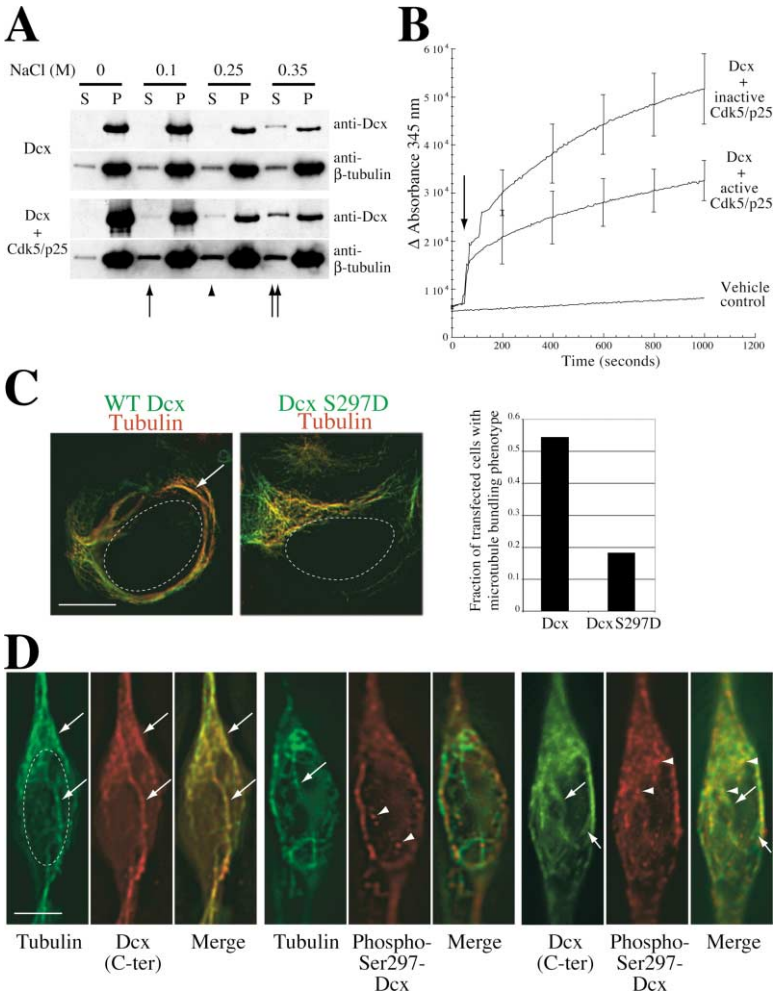


Figure 5. Phosphorylation of Dcx by Cdk5 Lowers Its Affinity for Microtubules and Its Effect on Polymerization

(A) Phosphorylation of wild-type Dcx by Cdk5/p25 impairs its co-assembly with microtubules. Dcx is present exclusively in the pellet (P) at 0 and 0.1 M NaCl, and a faint band first detectable in the supernatant (S) at 0.25 M NaCl (arrowhead). After Cdk5/p25 treatment, Dcx is detected in the pellet at 0.1 M NaCl (arrow), and a greater proportion is detected at each stepwise increase in NaCl concentration up to 0.35 M (double arrows).

(B) Dcx phosphorylated by Cdk5 has reduced effect on microtubule polymerization. Turbidimetric assays with phosphocellulose-purified tubulin using recombinant Dcx treated with either active Cdk5/p25 or heat-inactivated Cdk5/p25. Dcx was mixed in tubulin solution at time 50 s (arrow) and the turbidity measurement was recorded for 1000 s. Error bar indicates SEM.

(C) Mutant Dcx that mimic phosphorylation at S297 displayed impaired effect of microtubule polymerization when overexpressed. COS-7 cells were transfected with wild-type Dcx or S297D Dcx and immunostained with anti-Dcx and anti-tubulin antibodies. Over half of the cells transfected with wild-type Dcx displayed bundling and whirls of perinuclear microtubules, whereas fewer than 20% of cells transfected with S297D Dcx displayed this effect.

(D) Phospho-Ser297 Dcx localized punctate in cultured migrating neurons. Pan-Dcx strongly colocalizes with microtubules (arrows in left panels). Phospho-Ser297 antibody shows a shift in distribution away from microtubule bundles (arrow in tubulin-stained cell), with more punctate immunoreactivity (arrowheads). Costaining with anti-pan Dcx and anti-phospho-Ser297 demonstrates distinct pools of Dcx. Phospho-Ser297 immunoreactivity was void of smooth microtubular structures seen in pan-Dcx immunoreactivity (arrows) and was instead rather punctate (arrowheads). Merged image shows staining with anti-pan Dcx (green structures) that does not react with anti-phospho-Ser297 (arrows). Dashed circle indicates the nucleus. Scale bar equals 5 μm.

by introducing an aspartate (D) residue at site S297. Such S to D mutations have, in other proteins, been shown to mimic the effect of phosphorylation because of the neutral to basic change in charge (Egelhoff et al., 1993; Huang and Erikson, 1994; Leger et al., 1997). This construct (Dcx S297D) was tested for its ability to promote microtubule bundling in transfected COS-7 cells, a known effect of Dcx (Horesh et al., 1999). After 2 days in culture, cells were fixed and stained for Dcx and tubulin. Cells transfected with each construct were scored for the presence of a microtubule phenotype. Over half of the cells transfected with wild-type Dcx displayed bundling and whirls of microtubules encircling the nucleus, whereas fewer than 20% of cells transfected with the S297D mutant displayed this effect (Figure 5C). Therefore, conditions that mimic phosphorylation of Dcx at S297 result in impaired microtubule polymerizing activity.

In order to test for the differential affinity of phosphorylated Dcx versus total Dcx to microtubules in neurons, migrating cerebellar granule neurons in culture were immunostained with anti-pan Dcx antibody or anti-phospho-Ser297 antibody, together with anti-tyrosinated tubulin antibody to mark microtubules. Anti-pan Dcx antibody signal closely overlapped with tubulin. In the majority of cells, anti-phospho-Ser297 antibody showed more punctate immunoreactivity (Figure 5D). Phospho-Ser297 immunoreactivity was significantly more punctate and failed to label many of the microtubule structures that were visualized with the pan-Dcx antibody. Together, these data suggest that phosphorylated Dcx at Ser297 has a lower affinity for microtubules and impaired microtubule polymerizing activity both in a purified protein system and in cultured cells.

Mutation of the Ser297 Blocks the Normal Overexpression Effect of Dcx on Migration

We sought to test whether Ser297 was important for the function of Dcx in neuronal migration, but there are no published models to test for significance of mutations on the function of neuronal migration genes in migrating neurons. Additionally, among the amino acid substitution mutations that have been reported to date, a S297 mutation is not represented. However, we noted during the course of our studies that overexpression of wild-type Dcx in migrating neurons led to an increase in

migration in a cell-autonomous fashion that was highly reproducible, and we therefore used this assay to test the effect of amino acid substitution at S297.

The widely used reaggregate cerebellar granule neuron migration assay (Hatten, 1985) was used with retroviral transduction for overexpression of Dcx, S297A, or S297D mutant Dcx. Both Dcx 297A and 297D encode for stable proteins that colocalize with microtubules in transfected cells (data not shown), suggesting that there is unlikely to be a major effect of these mutations on protein folding. Retroviral constructs encoded wild-type or mutant *dcx* in the first cistron and GFP in the second cistron, allowing for identification of overexpressing neurons. Overexpression of Dcx using this method resulted in total Dcx protein levels approximately twice the wild-type levels (data not shown). After 12 hr of migration, a fraction of the neurons had migrated radially from the reaggregates, and the distance between each GFP-positive cell body and the edge of the reaggregate was measured, allowing for an integrated measurement of the rate of migration for each neuron.

Neurons transduced with GFP alone were positioned indistinguishably from untransduced neurons (data not shown), suggesting that retrovial transduction itself had no effect on migration. Neurons overexpressing wild-type Dcx displayed a rightward shift of migration bin distribution, indicating enhanced migration distances as a whole (Figure 6A). The average migration distance increased by 20 μm (34%) with Dcx overexpression ($p < 0.01$, Kruskal-Wallis nonparametric analysis; Glantz, 1996). In contrast, neurons overexpressing the S297A or S297D mutant Dcx displayed almost identical migration bin distribution to the GFP control ($p > 0.05$). There was no net enhancement in migration of either mutant ($p > 0.05$), suggesting that neither constitutive pseudo-dephosphorylation or pseudo-phosphorylation are sufficient for this effect of Dcx. It instead suggests that dynamic phosphorylation and dephosphorylation of Dcx may be critical for its effect on migration in migrating neurons.

Cdk5 Inhibition by Roscovitine Impairs the Dcx Overexpression Effect on Migration

In order to determine the functional relation between Dcx and Cdk5 on neuronal migration, the Dcx overexpression effect on migration was examined in the presence of roscovitine, the most selective Cdk5 inhibitor available. Roscovitine displays an IC_{50} of 200 nM for Cdk5, compared with IC_{50} of over 500 nM for cell cycle-related Cdks tested (Meijer et al., 1997). Roscovitine was added to the media to a final concentration of 20 μM (Niethammer et al., 2000) in migrating neurons and led to a leftward shift in bin distribution compared with untreated cells (Figure 6B). The average migration distance in 12 hr decreased by 10 μm (16%) from the control level ($p < 0.05$) (Figure 6B). This suggests Cdk5 inhibition leads to decreased migration in this assay.

We tested whether Dcx overexpression exerts the same positive effect on migration under the condition of Cdk5 inhibition to determine whether Dcx function is dependent on Cdk5 activity. The migration assay was performed on Dcx-overexpressing neurons in the presence of roscovitine. There was no increase in migration distances, indicating impaired effect of Dcx overexpression ($p > 0.05$ for comparison between GFP + roscovitine versus Dcx + roscovitine). Because pharmacological inhibition can block multiple enzymatic pathways, we repeated the experiment in the presence of kenpaullone, a mixed kinase inhibitor with an IC_{50} of 850 nM for Cdk5 versus 230 nM for GSK3β, and intermediate values for other Cdks (Zaharevitz et al., 1999). Published data suggests that effects blocked by both roscovitine and kenpaullone can be taken as specific for Cdks (Bain et al., 2003), and Cdk5 is likely the active Cdk in migrating neurons. Kenpaullone at 40 μM also abrogated the effect of Dcx overexpression on neuronal migration similar to roscovitine, although there appeared to be an additional toxic effect of the drug on the cells (data not shown). Thus, these data suggest that the effect of Dcx on neuronal migration is at least partly dependent on Cdk5 activity.

Discussion

Distinct but Similar Mechanism Underlying Migration Defects Caused by *DCX* Mutation in Human and *cdk5* Deletion in Mouse

The most noticeable similar features in *DCX* mutation and *cdk5* deletion are an upper thin cell-rich layer 2 and cell accumulation in the thick layer 4. In *cdk5* knockout mouse brain, this layer 2 was shown to arise from early cortical plate neurons (Gilmore et al., 1998). Subsequent neurons do not migrate over the preceding earlier born neurons and as a result accumulate inversely with regard to their birth date under the subplate, thus producing an extremely cell-rich layer 4. The precise cytoarchitectonic mechanism of lissencephaly due to *DCX* mutations is still unknown; however, *DCX* mutations give rise to another type of neuronal migration disorder in females, known as subcortical band heterotopia (SBH). The key feature of SBH is a band of heterotopic neurons in the subcortical white matter, suggesting that *DCX* mutations disable neuronal migration in what is thought to be the intermediate zone or subplate during development. The similarity in the cortex architectonics between *DCX* mutation and *cdk5* deletion suggested both may play critical roles in migration through embryonic telencephalon boundaries and these two proteins may interact functionally during this process.

Interestingly, there is another causative gene for lissencephaly in human, which is *LIS1* on chromosome 17p13.3. Mutations of *LIS1* lead to almost an identical neuronal migration defect phenotype as *DCX* mutations, and the LIS1-interacting protein NUDEL has been shown to be phosphorylated by Cdk5 (Niethammer et al., 2000; Sasaki et al., 2000), suggesting an additional intersection of pathways. It should be noted that despite the phenotypic similarity and these interactions, there are also clear differences in the cytoarchitectonic defect between murine *cdk5* knockout and the *dcx* or *lis1* mutants (Corbo et al., 2002; Gambello et al., 2003; Gilmore et al., 1998; Hirotsune et al., 1998; Ohshima et al., 1996).

Ser297 Is the Major Cdk5 Phosphorylation Site in Dcx

We identified that Ser297 is the major phosphorylation site by Cdk5. Phosphorylation of this site occurs in hu-

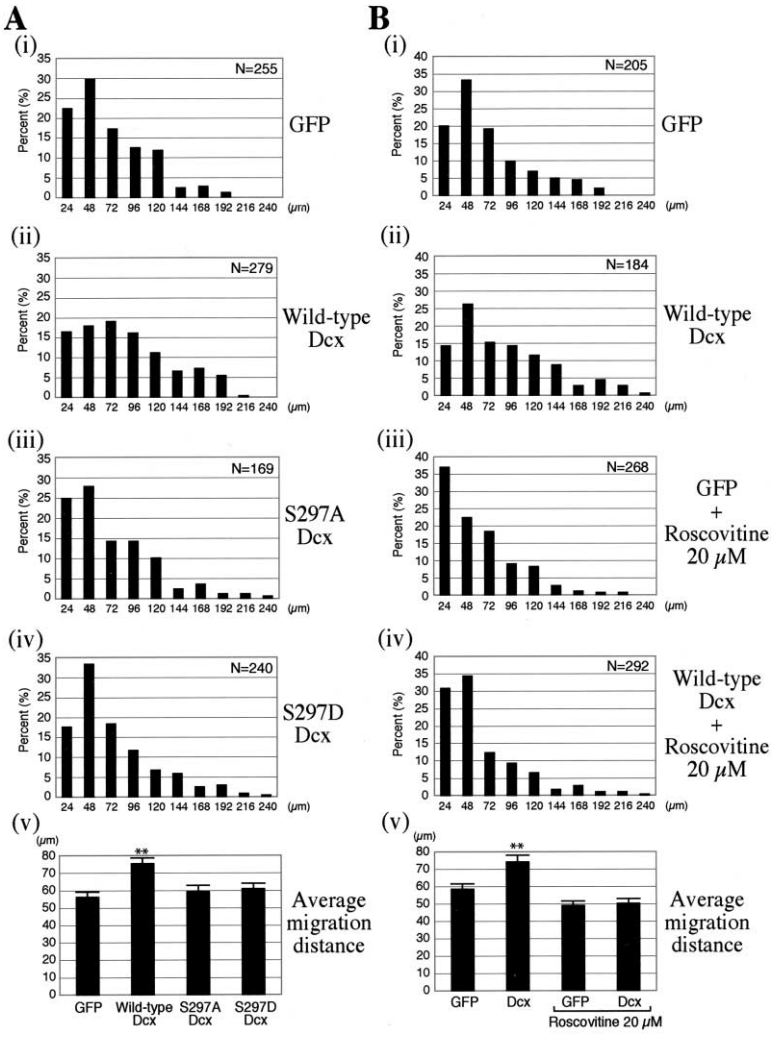


Figure 6. Mutation of the Ser297 Blocks the Normal Overexpression Effect of Dcx on Neuronal Migration in a Fashion Similar to Pharmacological Inhibition of Cdk5 Activity

(A) Migration distances (in μm) on the *x* axis, and percent of neurons in each bin along the *y* axis. N indicates number of neurons measured for each variable. Neurons transduced with Dcx + GFP display a shift in the bin distribution toward the right. The S297A or S297D mutant display similar migration bin distribution to the control. Data is summarized in (v).

(B) In the presence of 20 μM roscovitine, neurons transduced with GFP showed a leftward shift from the control (iii), and Dcx overexpression had no obvious effect on migration bin distribution or the average migration distance ($p > 0.05$). Error bars indicate SEM. ** indicates differs from GFP control at $p < 0.01$.

man as well, as phospho-Ser297 antibody reacted with human fetal brain lysates (T.T., F.F.S., and J.G.G., unpublished data). The Ser28 site was another candidate, based on x mutant analysis. We created a phospho-Ser28 antibody that reacted with recombinant Dcx phosphorylated in vitro by Cdk5/p25 but did not react with whole-brain lysates (unpublished data). Therefore, the S28 residue may be a substrate in vitro, but our best efforts failed to detect phosphorylation of S28 in vivo. The data together suggest that if Ser28 is phosphorylated, then other methods will be necessary to confirm this interaction.

Phosphorylation of Dcx by Cdk5 Likely Regulates Neuronal Migration through an Effect on Microtubule Dynamics

Phosphorylation of microtubule-associated proteins may be a general mechanism of regulating microtubule stability. The phosphorylation of tau reduces its affinity for microtubules and its ability to stabilize microtubules against nocodazole-induced depolymerization (Bramblett et al., 1993; Lindwall and Cole, 1984; Takahashi et al., 2003). Phosphorylation of MAP2 by protein kinase C reduces its ability to induce tubulin polymerization (Hoshi et al., 1988). Our data provide the first evidence for regulation of Dcx function by phosphorylation. We demonstrated that phosphorylation by Cdk5 reduced the amount of Dcx that co-assembles with microtubules, a measure of affinity, and reduced the polymerizing effect of Dcx, a measure of stabilization. Decreased affinity and stabilization effects of Dcx should destabilize microtubules, an effect that may lead to a more dynamic microtubule cytoskeleton. Neurons display two pools of microtubules, a cold-stable pool and a cold-labile pool (Brady et al., 1984), although the different functions that these two pools subserve has not been clarified. It is thought that negative regulation of MAPs using mechanisms such as phosphorylation may contribute to the function of the more labile pool of microtubules (Ebneth et al., 1999; Illenberger et al., 1996).

One apparent contradiction raised by our data was that Cdk5 phosphorylation of Dcx decreased its affinity for microtubules, yet we found that in cultured *cdk5*$^{-/-}$ neurons, Dcx was not as visible on fine perinuclear microtubules, despite the fact that Dcx should be hypophosphorylated in this state. One would have predicted that in the absence of *cdk5*, Dcx would have greater affinity to microtubules. Therefore, it appears that Dcx

phosphorylation is required for localization to these fine perinuclear microtubules. One potential explanation is that there might be other forces that select against incorporation of non-phospho Dcx (or other non-phospho MAPs) into these structures, which might render them more dynamic. Alternatively, non-phospho Dcx may have a higher affinity for more stable microtubules. Further study is in need to clarify phosphorylation effect of Dcx in different areas of the neuronal cytoplasm, especially perinuclear versus distal regions.

Defects in microtubule dynamics in connection with defects in nuclear migration were previously reported in other species. In *A. nidulans*, mutations in nuclear distribution genes, *nudA* or *nudF*, produce a nuclear migration defect, and the mutants have a lower frequency of microtubule catastrophe, shrinkage during catastrophe, and frequency of rescue (Han et al., 2001). In *S. cerevisiae*, astral microtubule dynamics are critical for nuclear migration to the bud neck (Yeh et al., 2000). In neuronal migration in mammals, a nuclear migration mechanism in fungus may have been recapitulated. An event such as neuronal migration, in which there is movement over hundreds of cell body distances, is likely to require significant reorganization of the cytoskeleton. Destabilization-stabilization cycles of microtubules by phosphorylation and dephosphorylation of MAPs may be important for dynamic changes in the cytoskeleton required for this movement. Phosphorylation of Dcx may be one factor that controls this dynamic regulation.

Colocalization of Dcx, Cdk5, and p35 Suggests a Role in Nuclear Translocation

The data together suggest that the regulation of Dcx may play a role in nuclear or somal translocation. We noted significant codistribution of Cdk5, p35, and Dcx in the region of the perinuclear soma, deficient localization of Dcx to fine perinuclear microtubules in *cdk5*$^{-/-}$ neurons, and the majority of phospho-Dcx immunostaining in the perinuclear/somal region in punctate structures. While firm data do not exist regarding a requirement for these factors in the nuclear/somal translocation phase of neuronal migration, other data is also consistent with this model. In *C. elegans*, the *dcx* orthologous gene *zyg-8* is required for proper spindle positioning during asymmetric division of one-cell stage embryos by promoting microtubule assembly during anaphase (Gönczy et al., 2001), an event that has many similarities to nuclear translocation. However, there was some Dcx, p35, Cdk5, as well as phospho-Ser297 signal detected in the leading and trailing processes of migrating neurons, and we cannot exclude the possibility that this interaction functions at these sites. It will be important to identify the cellular defect that underlies the neuronal migration phenotype in order to understand the function of Dcx more fully.

Effect of Dcx on Neuronal Migration Partly Depends on Cdk5

Together our data suggest that phosphorylation of Dcx Ser297 by Cdk5 is required for its normal activity in migration. We found that a 297A mutation blocked the overexpression effect of Dcx on migration, suggesting that Cdk5 phosphorylation at this site is necessary for its function. Countering these results was the finding that a 297D mutation also blocked the overexpression effect of Dcx on migration, suggesting that phosphorylation at this site is not sufficient for its function. It is possible that any mutation of S297 interferes with a critical protein interaction to inactivate Dcx. It is also possible that this D mutation does not mimic phosphorylation at this site, or that phosphorylation at this site is not directly related to migration. However, the other results, together with this data, favors the model in which it is the regulation of this phosphorylation that is critical for the function of Dcx.

Roscovitine is a potent and selective inhibitor of Cdk5 at the concentration used (Meijer et al., 1997), but it may inhibit the function of other kinases, particularly at higher concentrations (Bain et al., 2003). When used in conjunction with kenpaullone in this system, the results can be taken as specific for inhibition of Cdks. Both had negative effects on migration, but the similarities of their actions suggest an inhibition of Cdk5. In cells overexpressing Dcx, roscovitine and kenpaullone both had abrogating effects of Dcx overexpression. Together the data suggest that the overexpression effect of Dcx on migration depends on Cdk5 activity.

It should be noted that Gilmore et al. found no defect in cerebellar granule neuron migration in a cerebellar explant system from *cdk5*$^{-/-}$ mice (Gilmore and Herrup, 2001), whereas we observed defective cerebellar granule neuron migration following pharmacological blockade of Cdk5. One reason for this discrepancy could be the difference in the migration assay systems that were used. It does appear that *cdk5* is required for migration of granule neurons, as there is a striking cerebellar granule neuron phenotype in *cdk5*$^{-/-}$ mice (Ohshima et al., 1996) that is cell autonomous (Ohshima et al., 1999). To further clarify this point, we tested cerebellar granule neurons derived from E18 *cdk5*$^{+/+}$ and *cdk5*$^{-/-}$ mice in this assay and also found a defect in migration (unpublished observation). Therefore, *cdk5* appears to be required for cerebellar granule neuron migration in this in vitro system, although not in the explant system used.

It appears that there are kinases other than Cdk5 that may phosphorylate and regulate the function of Dcx, either at the 297 site or at other sites. We found evidence of residual Dcx phosphorylation in *cdk5*$^{-/-}$ brain, suggesting the presence of other kinases. Additionally, at longer exposures of the Western blotted with the phospho-Ser297-Dcx antibody, there were shadow bands that were evident in *cdk5*$^{-/-}$ brain, suggesting that other kinases may be capable of phosphorylating Dcx at this site, albeit with lower activity. Finally, in this issue, Schaar et al. (2004) report compelling evidence that MARK/PAR-1 regulate the function of Dcx through phosphorylation. These complementary studies show that regulation of Dcx function in neuronal migration is through several intersecting pathways.

Experimental Procedures

Animals

cdk5$^{+/-}$ mice were maintained in a mixed SV129/Bl6 background through intercrossing. Wild-type C57BL6 pregnant or P5 mice were obtained from Harlan (Indianapolis, IN). Animal work was carried out in compliance with the IACUC.

Immunoprecipitation and Western Blot Analysis

Cells or mouse brains were lysed with modified RIPA buffer with protease inhibitors (AEBSF, PMSF, aprotinin, leupeptin), treated with 1–4 μl CIP (10 U/μl), and incubated at 37°C for 1 hr. For immunoprecipitation, mouse telencephalon was homogenized in modified RIPA buffer and anti-Dcx (Gleeson et al., 1999) and anti-p35 (C-19, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies, or control rabbit serum was added and incubated for 1 hr, followed by addition of Protein A Sepharose beads (Pharmacia) for 30 min. Beads were washed with PBS four times and analyzed by Western blotting with anti-Dcx (1:5000) (Taylor et al., 2000).

Cdk5 Kinase Assay

Recombinant Dcx fragments were prepared as HIS_6-tagged proteins, concentrated to 2 mg/ml, and diluted to equimolar concentrations as described (Taylor et al., 2000). Recombinant GST-fusion Cdk5/p25 proteins were used at 1 mg/ml concentration. Kinase assay was performed as previously described (Niethammer et al., 2000).

In Situ Hybridization

S35-labeled antisense and sense riboprobes for *Dcx* were generated by in vitro transcription of pPCR-Script plasmid containing *Dcx* cDNA insert using T7 and T3 RNA polymerases. In situ hybridization on frozen sections was performed as previously described (Simmons et al., 1990).

Peptide Sample Preparation and MALDI-TOF MS

Three purified proteins, Dcx, Cdk5, and p35, were mixed in the kinase buffer to generate in vitro phosphorylated Dcx. Phosphorylation was controlled to one phosphate per Dcx, monitored by Cerenkov autoradiation counting. Phosphorylation was stopped by a short heat treatment. Protein mixtures were incubated with sequencing-grade trypsin (Promega, Madison, WI) at ratio of 50:1 (proteins:trypsin, w/w) at 37°C overnight. Tryptic peptides were dried in a SpeedVac concentrator (Thermo Savant, Holbrook, NY). The peptides were redissolved in a solution of 0.1% TFA (v/v) and 5% acetonitril (v/v), followed by a desalting step using ZipTip C18 tips (Millpore, Bedford, MA). Peptides bound to ZipTips were eluted with 2 μl of elution solution (10 mg/ml α-cyano-4-hydroxycinnamic acid, 50% acetonitril, and 0.1% TFA), and directly spotted onto a MALDI target plate. The MALDI-TOF MS spectra were obtained using a delayed-extraction reflectron time-of-flight mass spectrometer (Applied Biosystems, Framington, MA). Tryptic peptide table of Dcx, Cdk5, and p35 were generated in ProteinProspector (http://prospector.ucsf.edu/).

Site-Directed Mutagenesis

Site-directed mutagenesis was performed on the *Dcx* clone using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All mutagenized clones were sequenced across the entire open reading frame to eliminate PCR errors before use.

Cell Transfection

293T and COS-7 cells were transfected with the DNA construct of interest using SuperFect (Qiagen) according to the manufacturers recommendations.

Generation of Phospho-Specific Antibody

Phosphopeptide [KTSAK(pS)PGPM(Ahx)] was synthesized, purified, and conjugated to KLH. Two bleeds were combined and affinity-purified using positive and negative selection by Biosource International (Hopkinton, MA).

Primary Cortical Culture

Cortical neurons were isolated and cultured from E17 mouse brains as described (Huettner and Baughman, 1986; Zaman et al., 1999). After 24 hr of culture in 37°C, 5% CO_2 incubator, the cells were fixed and immunostained.

Immunofluorescence Microscopy and Quantitative Image Analysis

The antibodies used were anti-Cdk5 (C-8) (1:50), anti-p35 (N-20) (1:50), anti-Dcx (C-ter [Gleeson et al., 1999]) (1:300), anti-Dcx (C-18) (1:200) (Santa Cruz), and anti-tyrosinated-tubulin (YL 1/2) (Harlan) (1:200). Cerebellar granule neurons were examined using a Deltavision deconvolution imaging system (Applied Precision, Seattle, WA), and images were captured using 0.2 μm Z-steps, with 10 cycles of deconvolution using Softworx ver. 2.5 (Applied Precision). For quantitative analysis of Dcx binding to microtubules, fine perinuclear microtubules from wild-type and *cdk5*$^{-/-}$ neurons were identified by tubulin staining from deconvolved images using the Linescan feature in Metamorph 4.5. The pixel intensity along microtubules for both tubulin and Dcx was standardized by dividing by the average intensity for each signal across the entire image, in order to correct for variations in image acquisition. This ratio was averaged from three individual culture experiments.

Dcx Microtubule Co-Assembly Assay

Recombinant Dcx (0.5 μg) samples (wild-type Dcx unphosphorylated, wild-type Dcx phosphorylated by Cdk5/p25) were incubated with 80 μg tubulin in 60 μl PEM-GTP buffer (100 mM sodium PIPES [pH 6.6], 1 mM EGTA, 1 mM $MgSO_4$, 1 mM GTP) with NaCl in the presence of 10 μM taxol (Calbiochem) at 37°C for 45 min. Samples were centrifuged through a 10% sucrose cushion as described (Chang et al., 2003).

Turbidity Assay

Microtubule polymerization assays were performed as previously described (Gleeson et al., 1999; Taylor et al., 2000). Recombinant wild-type Dcx (5 μg) was incubated with 1.4 μg of either active or boiled inactive recombinant Cdk5/p25 in kinase buffer containing 50 μM ATP in a 10 μl reaction at 37°C for 3 hr. After recording turbidity, Dcx (5 μg) treated with either active or inactive Cdk5/p25 was mixed into the tubulin solution.

Retroviral Vector Constructs

Full-length N-terminal HIS-Xpress epitope tagged *DCX* (GenBank Index 2792349) in the KpnI site in pcDNA3.1 (Gleeson et al., 1999) were shuttled by restriction digestion with EcoRI and HindIII, followed by blunt end ligation into the EcoRI site in the first cistronic position of pCX-IEGFP (modified from pCXbsr by replacing bsr with EGFP) (Akagi et al., 2000) to create pCX-DCX-IEGFP constructs, followed by site-directed mutagenesis and sequencing.

Production of Recombinant Retroviruses

Recombinant ecotropic replication-incompetent Moloney murine leukemia virus-based retroviruses were produced according to protocols (Naviaux et al., 1996; Tomoda et al., 1999).

Reaggregate Neuronal Migration Assay

Cerebellar granule cells were isolated and cultured from P5 mice as described previously (Bix and Clark, 1998; Hatten, 1985). At the completion of the experiment, the images were analyzed to determine the position of each GFP-positive neuron relative to the edge of the reaggregate using the CALIPER feature of MetaMorph ver. 4.5. For Cdk5 inhibition, roscovitine or kenpaullone (Calbiochem) were added to the media immediately after the reaggregates were transferred on the coated slides.

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References

Akagi, T., Shishido, T., Murata, K., and Hanafusa, H. (2000). v-Crk activates the phosphoinositide 3-kinase/AKT pathway in transformation. Proc. Natl. Acad. Sci. USA *97*, 7290–7295.

Bai, J., Ramos, R.L., Ackman, J.B., Thomas, A.M., Lee, R.V., and LoTurco, J.J. (2003). RNAi reveals doublecortin is required for radial migration in rat neocortex. Nat. Neurosci. *6*, 1277–1283.

Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003). The specificities of protein kinase inhibitors: an update. Biochem. J. *371*, 199–204.

Bhaskar, K., Shareef, M.M., Sharma, V.M., Shetty, A.P., Ramamohan, Y., Pant, H.C., Raju, T.R., and Shetty, K.T. (2004). Co-purification and localization of Munc18–1 (p67) and Cdk5 with neuronal cytoskeletal proteins. Neurochem. Int. *44*, 35–44.

Bix, G.J., and Clark, G.D. (1998). Platelet-activating factor receptor stimulation disrupts neuronal migration In vitro. J. Neurosci. *18*, 307–318.

Brady, S.T., Tytell, M., and Lasek, R.J. (1984). Axonal tubulin and axonal microtubules: biochemical evidence for cold stability. J. Cell Biol. *99*, 1716–1724.

Bramblett, G.T., Goedert, M., Jakes, R., Merrick, S.E., Trojanowski, J.Q., and Lee, V.M. (1993). Abnormal tau phosphorylation at Ser396 in Alzheimer's disease recapitulates development and contributes to reduced microtubule binding. Neuron *10*, 1089–1099.

Caspi, M., Atlas, R., Kantor, A., Sapir, T., and Reiner, O. (2000). Interaction between LIS1 and doublecortin, two lissencephaly gene products. Hum. Mol. Genet. *9*, 2205–2213.

Chae, T., Kwon, Y.T., Bronson, R., Dikkes, P., Li, E., and Tsai, L.H. (1997). Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. Neuron *18*, 29–42.

Chang, L., Jones, Y., Ellisman, M.H., Goldstein, L.S., and Karin, M. (2003). JNK1 is required for maintenance of neuronal microtubules and controls phosphorylation of microtubule-associated proteins. Dev. Cell *4*, 521–533.

Corbo, J.C., Deuel, T.A., Long, J.M., LaPorte, P., Tsai, E., Wynshaw-Boris, A., and Walsh, C.A. (2002). Doublecortin is required in mice for lamination of the hippocampus but not the neocortex. J. Neurosci. *22*, 7548–7557.

Delalle, I., Bhide, P.G., Caviness, V.S., Jr., and Tsai, L.H. (1997). Temporal and spatial patterns of expression of p35, a regulatory subunit of cyclin-dependent kinase *5*, in the nervous system of the mouse. J. Neurocytol. *26*, 283–296.

Dobyns, W.B., Andermann, E., Andermann, F., Czapansky-Beilman, D., Dubeau, F., Dulac, O., Guerrini, R., Hirsch, B., Ledbetter, D.H., Lee, N.S., et al. (1996). X-linked malformations of neuronal migration. Neurology *47*, 331–339.

Ebneth, A., Drewes, G., Mandelkow, E.M., and Mandelkow, E. (1999). Phosphorylation of MAP2c and MAP4 by MARK kinases leads to the destabilization of microtubules in cells. Cell Motil. Cytoskeleton *44*, 209–224.

Egelhoff, T.T., Lee, R.J., and Spudich, J.A. (1993). Dictyostelium myosin heavy chain phosphorylation sites regulate myosin filament assembly and localization in vivo. Cell *75*, 363–371.

Francis, F., Koulakoff, A., Boucher, D., Chafey, P., Schaar, B., Vinet, M.C., Friocourt, G., McDonnell, N., Reiner, O., Kahn, A., et al. (1999). Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. Neuron *23*, 247–256.

Friocourt, G., Chafey, P., Billuart, P., Koulakoff, A., Vinet, M.C., Schaar, B.T., McConnell, S.K., Francis, F., and Chelly, J. (2001). Doublecortin interacts with mu subunits of clathrin adaptor complexes in the developing nervous system. Mol. Cell. Neurosci. *18*, 307–319.

Gambello, M.J., Darling, D.L., Yingling, J., Tanaka, T., Gleeson, J.G., and Wynshaw-Boris, A. (2003). Multiple dose-dependent effects of Lis1 on cerebral cortical development. J. Neurosci. *23*, 1719–1729.

Gilmore, E.C., and Herrup, K. (2001). Neocortical cell migration: GABAergic neurons and cells in layers I and VI move in a cyclin-dependent kinase 5-independent manner. J. Neurosci. *21*, 9690–9700.

Gilmore, E.C., Ohshima, T., Goffinet, A.M., Kulkarni, A.B., and Herrup, K. (1998). Cyclin-dependent kinase 5-deficient mice demonstrate novel developmental arrest in cerebral cortex. J. Neurosci. *18*, 6370–6377.

Glantz, S.A. (1996). Primer of Biostatistics, 4th edition (New York: McGraw-Hill).

Gleeson, J.G., and Walsh, C.A. (2000). Neuronal migration disorders: from genetic diseases to developmental mechanisms. Trends Neurosci. *23*, 352–359.

Gleeson, J.G., Lin, P.T., Flanagan, L.A., and Walsh, C.A. (1999). Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron *23*, 257–271.

Gönczy, P., Bellanger, J.-M., Kirkham, M., Pozniakowski, A., Baumer, K., Phillips, J.B., and Hyman, A.A. (2001). *zyg-8*, a gene required for spindle positioning in *C. elegans*, encodes a doublecortin-like kinase. Dev. Cell *1*, 363–375.

Gupta, A., Tsai, L.H., and Wynshaw-Boris, A. (2002). Life is a journey: a genetic look at neocortical development. Nat. Rev. Genet. *3*, 342–355.

Han, G., Liu, B., Zhang, J., Zuo, W., Morris, N.R., and Xiang, X. (2001). The *Aspergillus* cytoplasmic dynein heavy chain and NUDF localize to microtubule ends and affect microtubule dynamics. Curr. Biol. *11*, 719–724.

Hatten, M.E. (1985). Neuronal regulation of astroglial morphology and proliferation in vitro. J. Cell Biol. *100*, 384–396.

Hirotsune, S., Fleck, M.W., Gambello, M.J., Bix, G.J., Chen, A., Clark, G.D., Ledbetter, D.H., McBain, C.J., and Wynshaw-Boris, A. (1998). Graded reduction of Pafah1b1 (Lis1) activity results in neuronal migration defects and early embryonic lethality. Nat. Genet. *19*, 333–339.

Horesh, D., Sapir, T., Francis, F., Wolf, S.G., Caspi, M., Elbaum, M., Chelly, J., and Reiner, O. (1999). Doublecortin, a stabilizer of microtubules. Hum. Mol. Genet. *8*, 1599–1610.

Hoshi, M., Akiyama, T., Shinohara, Y., Miyata, Y., Ogawara, H., Nishida, E., and Sakai, H. (1988). Protein-kinase-C-catalyzed phosphorylation of the microtubule-binding domain of microtubule-associated protein 2 inhibits its ability to induce tubulin polymerization. Eur. J. Biochem. *174*, 225–230.

Huang, W., and Erikson, R.L. (1994). Constitutive activation of Mek1 by mutation of serine phosphorylation sites. Proc. Natl. Acad. Sci. USA *91*, 8960–8963.

Huettner, J.E., and Baughman, R.W. (1986). Primary culture of identified neurons from the visual cortex of postnatal rats. J. Neurosci. *6*, 3044–3060.

Illenberger, S., Drewes, G., Trinczek, B., Biernat, J., Meyer, H.E., Olmsted, J.B., Mandelkow, E.M., and Mandelkow, E. (1996). Phosphorylation of microtubule-associated proteins MAP2 and MAP4 by the protein kinase p110mark. Phosphorylation sites and regulation of microtubule dynamics. J. Biol. Chem. *271*, 10834–10843.

Keshvara, L., Magdaleno, S., Benhayon, D., and Curran, T. (2002). Cyclin-dependent kinase 5 phosphorylates disabled 1 independently of Reelin signaling. J. Neurosci. *22*, 4869–4877.

Kizhatil, K., Wu, Y.X., Sen, A., and Bennett, V. (2002). A new activity of doublecortin in recognition of the phospho-FIGQY tyrosine in the cytoplasmic domain of neurofascin. J. Neurosci. *22*, 7948–7958.

Ko, J., Humbert, S., Bronson, R.T., Takahashi, S., Kulkarni, A.B., Li, E., and Tsai, L.H. (2001). p35 and p39 are essential for cyclin-dependent kinase 5 function during neurodevelopment. J. Neurosci. *21*, 6758–6771.

Leger, J., Kempf, M., Lee, G., and Brandt, R. (1997). Conversion of

serine to aspartate imitates phosphorylation-induced changes in the structure and function of microtubule-associated protein tau. J. Biol. Chem. *272*, 8441–8446.

Lindwall, G., and Cole, R.D. (1984). Phosphorylation affects the ability of tau protein to promote microtubule assembly. J. Biol. Chem. *259*, 5301–5305.

Matsushita, M., Matsui, H., Itano, T., Tomizawa, K., Tokuda, M., Suwaki, H., Wang, J.H., and Hatase, O. (1995). Developmental changes of cyclin dependent kinase 5 subcellular localization in the rat cerebellum. Neuroreport *6*, 1267–1270.

Meijer, L., Borgne, A., Mulner, O., Chong, J.P., Blow, J.J., Inagaki, N., Inagaki, M., Delcros, J.G., and Moulinoux, J.P. (1997). Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. Eur. J. Biochem. *243*, 527–536.

Naviaux, R.K., Costanzi, E., Haas, M., and Verma, I.M. (1996). The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. J. Virol. *70*, 5701–5705.

Niethammer, M., Smith, D.S., Ayala, R., Peng, J., Ko, J., Lee, M.S., Morabito, M., and Tsai, L.H. (2000). NUDEL is a novel Cdk5 substrate that associates with LIS1 and cytoplasmic dynein. Neuron *28*, 697–711.

Nikolic, M., Dudek, H., Kwon, Y.T., Ramos, Y.F., and Tsai, L.H. (1996). The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. Genes Dev. *10*, 816–825.

Nikolic, M., Chou, M.M., Lu, W., Mayer, B.J., and Tsai, L.H. (1998). The p35/Cdk5 kinase is a neuron-specific Rac effector that inhibits Pak1 activity. Nature *395*, 194–198.

Ohshima, T., Ward, J.M., Huh, C.G., Longenecker, G., Veeranna, Pant, H.C., Brady, R.O., Martin, L.J., and Kulkarni, A.B. (1996). Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. Proc. Natl. Acad. Sci. USA *93*, 11173–11178.

Ohshima, T., Gilmore, E.C., Longenecker, G., Jacobowitz, D.M., Brady, R.O., Herrup, K., and Kulkarni, A.B. (1999). Migration defects of cdk5(−/−) neurons in the developing cerebellum is cell autonomous. J. Neurosci. *19*, 6017–6026.

Patrick, G.N., Zukerberg, L., Nikolic, M., de la Monte, S., Dikkes, P., and Tsai, L.H. (1999). Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature *402*, 615–622.

Patzke, H., Maddineni, U., Ayala, R., Morabito, M., Volker, J., Dikkes, P., Ahlijanian, M.K., and Tsai, L.H. (2003). Partial rescue of the p35−/− brain phenotype by low expression of a neuronal-specific enolase p25 transgene. J. Neurosci. *23*, 2769–2778.

Sapir, T., Horesh, D., Caspi, M., Atlas, R., Burgess, H.A., Wolf, S.G., Francis, F., Chelly, J., Elbaum, M., Pietrokovski, S., and Reiner, O. (2000). Doublecortin mutations cluster in evolutionarily conserved functional domains. Hum. Mol. Genet. *9*, 703–712.

Sasaki, S., Shionoya, A., Ishida, M., Gambello, M.J., Yingling, J., Wynshaw-Boris, A., and Hirotsue, S. (2000). A LIS1/NUDEL/cytoplasmic dynein heavy chain complex in the developing and adult nervous system. Neuron *28*, 681–696.

Schaar, B.T., Kinoshita, K., and McConnell, S.K. (2004). Doublecortin microtubule affinity is regulated by a balance of kinase and phosphatase activity at the leading edge of migrating neurons. Neuron *42*, this issue, 203–213.

Simmons, D.M., Voss, J.W., Ingraham, H.A., Holloway, J.M., Broide, R.S., Rosenfeld, M.G., and Swanson, L.W. (1990). Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. Genes Dev. *4*, 695–711.

Songyang, Z., Lu, K.P., Kwon, Y.T., Tsai, L.H., Filhol, O., Cochet, C., Brickey, D.A., Soderling, T.R., Bartleson, C., Graves, D.J., et al. (1996). A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, CDK5, and Erk1. Mol. Cell. Biol. *16*, 6486–6493.

Takahashi, S., Saito, T., Hisanaga, S., Pant, H.C., and Kulkarni, A.B. (2003). Tau phosphorylation by cyclin-dependent kinase 5/p39 during brain development reduces its affinity for microtubules. J. Biol. Chem. *278*, 10506–10515.

Tanaka, T., Veeranna, Ohshima, T., Rajan, P., Amin, N.D., Cho, A., Sreenath, T., Pant, H.C., Brady, R.O., and Kulkarni, A.B. (2001). Neuronal cyclin-dependent kinase 5 activity is critical for survival. J. Neurosci. *21*, 550–558.

Taylor, K.R., Holzer, A.K., Bazan, J.F., Walsh, C.A., and Gleeson, J.G. (2000). Patient mutations in doublecortin define a repeated tubulin-binding domain. J. Biol. Chem. *275*, 34442–34450.

Tomizawa, K., Matsui, H., Matsushita, M., Lew, J., Tokuda, M., Itano, T., Konishi, R., Wang, J.H., and Hatase, O. (1996). Localization and developmental changes in the neuron-specific cyclin-dependent kinase 5 activator (p35nck5a) in the rat brain. Neuroscience *74*, 519–529.

Tomoda, T., Bhatt, R.S., Kuroyanagi, H., Shirasawa, T., and Hatten, M.E. (1999). A mouse serine/threonine kinase homologous to C. elegans UNC51 functions in parallel fiber formation of cerebellar granule neurons. Neuron *24*, 833–846.

Tsai, L.H., Takahashi, T., Caviness, V.S., Jr., and Harlow, E. (1993). Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. Development *119*, 1029–1040.

Vallee, R.B. (1986). Reversible assembly purification of microtubules without assembly-promoting agents and further purification of tubulin, microtubule-associated proteins, and MAP fragments. In Methods Enzymol., pp. 89–104.

Yeh, E., Yang, C., Chin, E., Maddox, P., Salmon, E.D., Lew, D.J., and Bloom, K. (2000). Dynamic positioning of mitotic spindles in yeast: role of microtubule motors and cortical determinants. Mol. Biol. Cell *11*, 3949–3961.

Zaharevitz, D.W., Gussio, R., Leost, M., Senderowicz, A.M., Lahusen, T., Kunick, C., Meijer, L., and Sausville, E.A. (1999). Discovery and initial characterization of the paullones, a novel class of small-molecule inhibitors of cyclin-dependent kinases. Cancer Res. *59*, 2566–2569.

Zaman, K., Ryu, H., Hall, D., O'Donovan, K., Lin, K.I., Miller, M.P., Marquis, J.C., Baraban, J.M., Semenza, G.L., and Ratan, R.R. (1999). Protection from oxidative stress-induced apoptosis in cortical neuronal cultures by iron chelators is associated with enhanced DNA binding of hypoxia-inducible factor-1 and ATF-1/CREB and increased expression of glycolytic enzymes, p21(waf1/cip1), and erythropoietin. J. Neurosci. *19*, 9821–9830.